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EFFECT OF TUBOCURARINE ON ACTION POTENTIALS IN NORMAL AND DENERVATED SKELETAL MUSCLE

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One of the changes known to occur in striated muscle fibres following denervation is a great increase in membrane area showing sensitivity to acetylcholine (ACh) (Ginetzinsky & Shamarina, 1942; Axelsson & Thesleff, 1959; Miledi, 1960*a*). In some cases, after several weeks' denervation, the whole surface may be nearly as sensitive as the subsynaptic membrane of intact fibres. Several lines of evidence suggest that the properties of the receptor sites responsible for ACh sensitivity are very similar if not identical to those found at the end-plate of intact muscle (Axelsson & Thesleff, 1959; Klaus, Kuschinsky, Lüllman & Muscholl, 1959; Miledi, 1960*b*; Jenkinson, 1960; Bhoola & Schachter, 1961). These sites may develop only after denervation, or they may be present throughout the membrane of normal muscle and be 'unmasked' following denervation. The latter alternative is preferred by those who suggest that ACh receptors play an important role in impulse conduction (Rothenberg, Sprinson & Nachmansohn, 1948; Hinterbuchner & Nachmansohn, 1960). If in fact these receptor sites are simply made accessible to drugs following denervation and if they are involved in the electrical activity of the muscle membrane, then one might expect that curarization of the denervated muscle would noticeably affect characteristics of the fibre membrane, at least during activity. This paper describes the results of experiments designed to look for such effects.

METHODS

Frog (*Rana temporaria*) sartorius muscles were dissected and mounted (deep surface up) on the arched Perspex floor of the recording chamber. They were stretched to approximately the normal, *in vivo*, length. The bathing solution had the following composition: NaCl, 116 mM; KCl, 2 mM; CaCl, 1·8 mM.

Denervation was carried out by removal of approximately 1 cm of the sciatic nerve at the level of the pelvis, and this was repeated 4–5 weeks later when a longer period of

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denervation was desired. Most frogs were used 1–2 months after denervation. The ACh sensitivity of denervated fibres was tested by ionophoretic application of ACh near the pelvic end of the muscle in the manner described by del Castillo & Katz (1955) and Miledi (1960*a*). At the same time the sensitivity of the normal end-plate membrane was determined in the control muscle. In most cases the denervated membrane was 10–1000 times less sensitive than the end-plate membrane.

Records were taken with 3 M-KCl-filled micropipettes of about 5–50 MΩ resistance, inserted 5–8 mm from the pelvic end of the fibre. The fibre was then stimulated individually by a short (0·25 msec) current pulse passed through a pore electrode (a broken microelectrode filled with Ringer's solution) placed on the fibre surface 1·5–3 mm nearer the pelvic end. In a few cases a second electrode was inserted into the fibre for intracellular stimulation and measurement of membrane characteristics. The input capacitance of the system was approximately 1·5 pF. Frequent checks were made of the rise-time characteristics of the recording system to ensure an accurate representation of the spike parameters under study.

D-Tubocurarine chloride (DTC) (Burroughs-Wellcome, crystalline) was applied to the whole muscle and, unless otherwise noted in the text, it was allowed to act for at least ½ hr before recording was resumed. The potency of the DTC was checked by its effectiveness in blocking neuromuscular transmission. DTC 5×10^{-6} M placed in the bath blocked all visible contraction in response to nerve stimulation within 10 min. In all cases control records were taken before and approximately 1 hr after exposure to the drug. Usually 10–15 fibres were used in each experimental condition, previously unused fibres being selected when possible. If fibres had to be used again, the results were accepted only if resting potentials and spike amplitudes were maintained at or above a criterion level (usually 85 and 110 mV, respectively). On a few occasions individual fibres were identified and studied in each experimental condition. Although most of these deteriorated during the experiment because of injury, a few retained a high resting potential and gave large spikes, and so could be used individually to show drug effects.

The spike parameters measured were: resting potential (RP), rise time between 50 % and peak amplitude, fall time between the peak and 50 % amplitude, total height, and maximum rates of rise and fall (MRR and MRF, measured after differentiation of the spike with a circuit having a measured time constant of 20 μsec and a calibrated linear response between 25 and 500 V/sec).

In other experiments, intercostal muscles of young (100–200 g) albino rats were used at room temperature. The bathing solution was that described by Liley (1956) and was kept oxygenated by direct bubbling with a 95 % O_2, 5 % CO_2 gas mixture, or by constant slow perfusion (3–4 ml./min) with vigorously bubbled solution. Recordings were made intracellularly in a part of the fibre well removed from the end-plate region. Intercostal muscles that had been denervated by simple cutting of a rib and its underlying nerve were also tested, usually within 2–3 weeks of denervation. But the resting potentials and fibre diameters had so decreased in that time that recorded spikes proved highly variable and quite unlike control spikes.

The temperature was maintained within 1–2° during any given experiment, but varied from day to day with room temperature, which ranged from approximately 17 to 22° C.

RESULTS

Frog sartorius fibres

Eight denervated muscles were studied that were very sensitive to ACh over their entire length. The sensitivity near the pelvic end was 0·5 to 60 mV membrane depolarization/10^{-9} C of charge passed, i.e. not less

than 1/100 of the maximum sensitivity found at normal intact end-plates (Miledi, 1960*a*). In these frogs, the action potentials from the denervated and control sartorius fibres (70–90 of each) showed no clearly significant differences, a result in agreement with that of Levine (1961). The average values for resting potential and various spike parameters are shown in the first two columns of Table 1.

TABLE 1. Average values of spike parameters of Control (C) and denervated (D) muscles before exposure to curare, in 10^{-4} and 10^{-3} M-DTC, and approximately 1 hr after removal of curare (± S.E.M.)

	Initial		10^{-4} M		10^{-3} M		Recovery	
	C	D	C	D	C	D	C	D
RP (mV)	90 ± 0·7	88·6 ± 0·6	91 ± 1	87 ± 1	90 ± 1	90 ± 1	90 ± 1	90 ± 1
Spike ampl. (mV)	129 ± 1·5	129 ± 1·7	130 ± 2·5	130 ± 4	124 ± 2·5	126 ± 2·5	126 ± 5	128 ± 2·5
Rise time* (msec)	0·38 ± 0·02	0·39 ± 0·03	0·39 ± 0·01	0·39 ± 0·01	0·44 ± 0·03	0·41 ± 0·02	0·41 ± 0·03	0·44 ± 0·03
Fall time† (msec)	1·25 ± 0·13	1·32 ± 0·09	1·86 ± 0·25	1·83 ± 0·24	2·04 ± 0·32	2·0 ± 0·17	1·41 ± 0·06	1·5 ± 0·08
MRR (mV/msec)	345 ± 17	354 ± 27	356 ± 14	343 ± 14	286 ± 17	318 ± 11	296 ± 17	332 ± 9
MRF (mV/msec)	61 ± 6·2	52 ± 3·1	44 ± 3	40 ± 2·5	45 ± 6	38 ± 4	49 ± 2·5	46 ± 2·5

* From 50% depolarization to the peak of the action potential.
† From the peak of the action potential to 50% repolarization.

TABLE 2. Average values of the measured spike parameters and their relative values in several concentrations of DTC (± S.E.M.)

	Initial value	5×10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	Recovery‡
RP	88·2 ± 0·7 mV	1·01 ± 0·01	1·01 ± 0·01	0·99 ± 0·01	1·00 ± 0·01	*0·79 ± 0·06*†	1·00 ± 0·01
Spike height	126 ± 0·7 mV	1·00 ± 0·01	1·00 ± 0·01	1·00 ± 0·01	0·97 ± 0·01	*0·74 ± 0·06*	0·98 ± 0·01
50% rise time	0·38 ± 0·02 msec	1·10 ± 0·06	1·13 ± 0·15	1·10 ± 0·02	*1·21 ± 0·05*	*1·91 ± 0·24*	1·08 ± 0·04
MRR	312 ± 11 mV/sec	0·99 ± 0·09	0·96 ± 0·06	0·95 ± 0·04	*0·81 ± 0·03*	*0·35**	0·89 ± 0·03
50% fall time	1·15 ± 0·04 msec	1·01 ± 0·04	*1·18 ± 0·08*	*1·43 ± 0·05*	*1·60 ± 0·18*	*3·09 ± 0·78*	1·14 ± 0·04
MRF	69 ± M mV/msec	0·99 ± 0·06	Not recorded	*0·71 ± 0·05*	*0·69 ± 0·06*	*0·26**	0·83 ± 0·04

* Data from single experiments.
† Data from only those fibres used in measurement of spike parameters.
‡ Recovery data was normally taken after 1 hr of washing. These figures refer to recovery after any of the curare concentrations used (see text).
Italic: significantly affected by DTC.

Also shown in Table 1 is the effect of 10^{-4} and 10^{-3} M-tubocurarine chloride (DTC) on these denervated muscles and their normal partners. In this respect also, there were no significant differences between the normal and sensitized fibres, although curare had a clear-cut effect on both: a lengthening of the falling phase of the spike (and a decrease in MRF) with little or no change in the other measured parameters. Since it seemed obvious that normal and denervated fibres were not differently affected, more extensive measurements were made on normally innervated muscle. In approximately 30 experiments, 4 trials were made with 5×10^{-6} M-DTC, 8 with 10^{-5}, 21 with 10^{-4}, 8 with 10^{-3}, and 2 with 10^{-2} M-DTC. The results of these experiments are shown in Table 2.

Resting potentials were unchanged after as long as 2 hr in 10^{-3} M-DTC. In 10^{-2} M-curare, the average RP fell to 59 mV (57 fibres) in 15 min,

showing that curare, like some local anaesthetics (e.g. Friebel, Karzel & Draper, 1960; Falk, 1961) causes depolarization at a sufficiently high concentration.

Total spike amplitude remained essentially unchanged at 10^{-3} M, but dropped to around 75% after $\frac{1}{2}$ hr in 10^{-2} M-DTC. A few experiments suggested a slight lengthening effect of 10^{-5} and 10^{-4} M-DTC on the rise time of the action potential, but this was not apparent in most experiments until the concentration reached 10^{-3} M (average 20% increase). At 10^{-2} M, the rise time was increased by 90% on the average. The MRR was also affected only at high concentration of DTC.

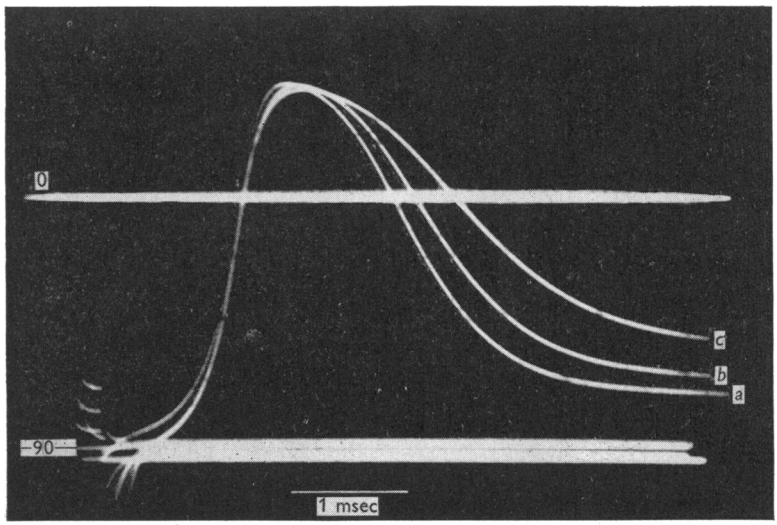

Fig. 1. Sample record showing the superimposed action potentials of a single frog sartorius fibre in normal Ringer's solution (*a*), and in 10^{-5} M (*b*) and 10^{-4} M-DTC (*c*).

In contrast to the parameters mentioned above, the repolarization phase of the action potential was comparatively sensitive to curare. Figure 1 illustrates the characteristic lengthening induced by concentrations of 10^{-5} and 10^{-4} M-DTC on the same single fibre. An increase in negative after-potential accompanied the change in fall time, as may also be seen in this figure.

The 'threshold' for this lengthening effect of curare is at approximately 5×10^{-6} M, at which concentration one of the four muscles tested was clearly affected. At 10^{-5} M fall time was prolonged by approximately 20% (range 90–136%); at 10^{-4} M, by 40% (range 107–194%); and at 10^{-3} M, by 60% (range 126–276%). At 10^{-2} M, depolarization of the fibre

and reduction of spike amplitude magnified the effect and many fibres gave highly atypical spikes or none at all. In fibres which still gave a characteristic propagated spike, the duration was increased by an average 200 %. The effect was entirely reversible even from this concentration.

Any given curare concentration appeared to reach its maximum effect within approximately 15 min, and no consistent change was found in the next 1–1·5 hr. Recovery was much slower and followed a roughly exponential time course, but reached completion whenever sufficient time was allowed. After high concentrations of curare this often required 1·5–2 hr. Since most measurements were made after only 1 hr of washing, the average values given for 'recovered' fibres in Table 2 show incomplete reversal of the effects of curare. Also complicating these measurements was the possibility of a change in spike parameters with time. In ten control experiments records taken from a muscle before and after 4–7 hr in Ringer's solution were compared. In none of these did RP or spike amplitude vary by as much as 10 %, and in half the experiments there was also no significant change in any of the other parameters. The remainder did show changes with time as large as 20–30 %, but not in a consistent way. In three cases the fall time increased; in two it decreased. Hence, in the average of a large number of experiments, it seems unlikely that time-dependent shifts in value are of critical importance.

Curare has been found to compete with ACh for receptors not only at the end-plate (cf. Jenkinson, 1960) but also over the whole membrane of denervated fibres (Axelsson & Thesleff, 1959). Hence it seemed of interest to determine whether ACh (or, in our experiments, carbachol) would compete with curare for the sites involved in the lengthening of the action potential. In only two of five experiments did this seem to be the case. In one of these, 10^{-3} M-carbachol reduced spike fall time from 134 to 117 % in 10^{-4} M-DTC, from 219 to 173 % in 10^{-3} M-DTC. In the other, 10^{-3} M-carbachol reduced the effect of 2×10^{-4} curare by about 40 %. In the remaining experiments, carbachol, even at 10^{-2} M concentration, did not reduce the effect of curare, but appeared to interact with curare somehow, since spike durations in curare *after* removal of carbachol were uniformly longer (average 15 %) than in the same curare concentration before carbachol. By itself, carbachol in concentrations as high as 10^{-2} M had no apparent lengthening effect on the action potential of fibres that had recovered from the initial depolarization.

Rat intercostal muscle

Since normal rat muscle appears to have no detectable ACh receptors in non-end plate regions, yet becomes more highly sensitive following denervation than does frog muscle (Miledi, 1962), attempts were made to

assess the effects of curare on normal and denervated rat intercostal fibres. Unfortunately, denervated fibres had resting potentials of 50–70 mV or less and gave very variable spikes, none of which were comparable to those of normal fibres. Any effects of curare were small compared to this variability. It should be noted, however, that denervated fibres did give conducted action potentials in 10^{-3} M-DTC.

Normal fibres showed a lengthening of the action potential in curare, with no evident effect on the other parameters measured; but sensitivity to curare was less than in frog muscle. The 'threshold' was at approximately 10^{-4} M-DTC, and only four of six experiments with 10^{-3} M showed a clear lengthening of fall time (average 15 %).

DISCUSSION

Our experiments provide no evidence for the hypothesis that the ACh-sensitive sites which appear following denervation are involved in impulse conduction. Curarization of the muscle does affect the action potential, but its action in all but extremely high concentrations is confined to the repolarization phase, which is not critical to the propagation of an impulse. Moreover, the lengthening effect of curare on action potentials in denervated muscle appears to be indistinguishable from its effect on innervated muscle, and is found even in innervated rat muscle which shows no detectable response to ACh applied locally away from the end-plate (Miledi, 1962). Hence it seems most unlikely that the lengthening action is mediated through the same receptors that react with ACh to produce depolarization. Curare *also* reacts with these ACh receptors, since it blocks the depolarizing effect of ACh on denervated muscle; but the membrane components responsible for slowing repolarization are probably quite separate and not affected by denervation. The observation that carbachol sometimes reduces the curare effect suggests that ACh can also react with the sites involved in the prolongation of the action potential.

The presence of more than one kind of reactive sites throughout the muscle membrane must be viewed as a considerable obstacle to isolation of a specific 'ACh-receptor substance'. Although curare blocks the effect of ACh at the end-plate in a lower concentration than is required to produce lengthening of the action potential, the receptor sites responsible for the lengthening of the action potential, which are presumably spread over the whole muscle surface, may preponderate.

The action of curare described in this paper is similar in many respects to that of several other substances, including tetraethylammonium chloride (TEA) (Hagiwara & Watanabe, 1955), caffeine, quinine, cocaine and procaine (Etzensperger, 1957; Falk, 1961). However, none of these

is effective at as low a concentration as curare and some, at least, cause a depolarization with time that is not observed with concentrations of DTC having a comparable lengthening effect.

Previous investigations (Rushton, 1933; Kuffler, 1945; del Castillo & Katz, 1957) have shown that curare, in neuromuscular blocking concentrations, has no effect on the electrical properties of the resting muscle membrane. Our results, which concern the effect of higher curare concentrations on the active fibre membrane, are not inconsistent with this finding. The fact that fall time of the action potential is much more strikingly affected than rise time suggests that curare may selectively influence K permeability in the electrically active membrane.

SUMMARY

1. Action potentials have been recorded intracellularly in normal and denervated frog sartorius fibres and in rat intercostal fibres. The effects of D-tubocurarine chloride (DTC) were studied.

2. There were no significant differences between the action potentials of normal and denervated frog fibres, either in Ringer's solution or in curare. But an action of curare was observed under both conditions.

3. A concentration of DTC greater than 5×10^{-6} M caused a just-detectable slowing in the repolarization phase of the action potential with no change in the other parameters. DTC 10^{-5} M caused an average 20 % lengthening; 10^{-4} M, 40 % and 10^{-3} M, 60 %.

4. DTC 10^{-3} M sometimes, and 10^{-2} M always, increased the rise time and decreased the amplitude of the action potential, but fibres still conducted impulses in 10^{-2} M-DTC. These effects were all fully reversible.

5. Carbachol 10^{-3} M appeared in some cases to interact with curare, decreasing its effect on the duration of the action potential or sensitizing the muscle membrane to subsequent action of curare. Carbachol 10^{-3} or 10^{-2}, by itself, had no effect on the action potential.

6. The action potentials of rat intercostal fibres were similarly affected by curare, but 10^{-4} M or higher concentrations were required.

7. There seems to be no relation between the ACh-sensitive sites that appear over the whole surface of denervated muscle and the sites involved in the lengthening effect of curare. Neither type of receptor appears to be involved in impulse conduction.

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